Long-term expression pattern of melanocyte markers in light- and dark-pigmented dermo-epidermal cultured human skin substitutes

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Abstract: PURPOSE: Transplantation of pigmented tissue-engineered human autologous skin substitutes represents a promising procedure to cover skin defects. We have already demonstrated that we can restore the patient's native light or dark skin color by adding melanocytes to our dermo-epidermal skin analogs. In this long-term study, we investigated if melanocytes in our skin substitutes continue to express markers as BCL2, SOX9, and MITF, known to be involved in survival, differentiation, and function of melanocytes. METHODS: Human epidermal melanocytes and keratinocytes, as well as dermal fibroblasts from light- and dark-pigmented skin biopsies were isolated and cultured. Bovine collagen hydrogels containing fibroblasts were prepared, and melanocytes and keratinocytes were seeded in a 1:5 ratio onto the gels. Pigmented dermo-epidermal skin substitutes were transplanted onto full-thickness wounds of immuno-incompetent rats and analyzed for the expression of melanocyte markers after 15 weeks. RESULTS: Employing immunofluorescence staining techniques, we observed that our light and dark dermo-epidermal skin substitutes expressed the same typical melanocyte markers including BCL2, SOX9, and MITF 15 weeks after transplantation as normal human light and dark skin. CONCLUSIONS: These data suggest that, even in the long run, our light and dark dermo-epidermal tissue-engineered skin substitutes contain melanocytes that display a characteristic expression pattern as seen in normal pigmented human skin. These findings have crucial clinical implications as such grafts transplanted onto patients should warrant physiological numbers, distribution, and function of melanocytes.

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ORIGINAL ARTICLE

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Abstract
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Methods Human epidermal melanocytes and keratinocytes, as well as dermal fibroblasts from light- and dark-pigmented skin biopsies were isolated and cultured. Bovine collagen hydrogels containing fibroblasts were prepared, and melanocytes and keratinocytes were seeded in a 1:5 ratio onto the gels. Pigmented dermo-epidermal skin substitutes were transplanted onto full-thickness wounds of immuno-incompetent rats and analyzed for the expression of melanocyte markers after 15 weeks.
Results Employing immunofluorescence staining techniques, we observed that our light and dark dermo-epidermal skin substitutes expressed the same typical melanocyte markers including BCL2, SOX9, and MITF 15 weeks after transplantation as normal human light and dark skin.
Conclusions These data suggest that, even in the long run, our light and dark dermo-epidermal tissue-engineered skin substitutes contain melanocytes that display a characteristic expression pattern as seen in normal pigmented human skin. These findings have crucial clinical implications as such grafts transplanted onto patients should warrant physiological numbers, distribution, and function of melanocytes.

Keywords Melanocytes · Human pigmented dermo-epidermal skin substitutes · Tissue engineering

Introduction

The application of split-thickness skin grafts still is the standard method to cover full-thickness skin defects resulting from excisions of large burn injuries, scar revisions, and giant congenital nevi [1, 2]. Obviously, the use of split-thickness skin is limited by the amount of donor sites available.

Laboratory cultured epidermal autografts (CEA) consisting of only keratinocytes were the first tissue engineering attempts to overcome this limitation and to provide enough autologous skin for the patient [3]. Often, the functional and esthetic outcomes after CEA application were unsatisfying [4]. To improve tissue-engineered skin, dermo-epidermal skin substitutes were introduced, containing epithelial cells and mesenchymal fibroblasts [5].

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Over the last 15 years, we have been developing dermo-epidermal skin substitutes with the goal to produce large, near-natural skin analogs to be applied clinically [6, 7]. In particular, we wanted to integrate melanocytes in our dermo-epidermal skin substitutes to produce physiologically pigmented constructs. Clearly, the absence or irregular distribution of melanocytes in skin substitutes makes the skin vulnerable with regard to UV irradiation and leads to disfiguring hypo- or hyperpigmented patches after transplantation [8].

We recently described the fabrication of such light- and dark-pigmented dermo-epidermal skin substitutes consisting of human keratinocytes, melanocytes, and fibroblasts [9]. These skin substitutes were transplanted in a long-term assay for 15 weeks and displayed the same light or dark skin color as the donor skin.

In the present experimental study, we wanted to investigate the presence and the expression pattern of typical described melanocyte markers in our pigmented dermo-epidermal skin substitutes (pigmentDESS) that are known to be involved in melanocyte survival, proliferation, differentiation, and function.

Materials and methods

Shortly, we transplanted light and dark pigmentDESS containing human epidermal melanocytes and keratinocytes, as well as dermal fibroblasts, isolated and cultured from light (Fitzpatrick skin type I–II) and dark (Fitzpatrick skin type V) skin samples. 15 weeks after transplantation, we investigated the pigmentDESS for the expression of the anti-apoptotic marker BCL2, the pigmentation-regulating factors SOX9 and MITF, and the survival and differentiation marker SOX10.

Human skin specimens

The study was conducted according to the Declaration of Helsinki and with permission by the Ethic Commission of the Canton Zurich. Informed consent for skin sampling was given by parents or patients. Human foreskin samples from patients ranging in age between one and 16 years were used.

Isolation and culturing of primary human cells

The cells were isolated from light (Fitzpatrick skin type I–II) and dark (Fitzpatrick skin type V) foreskin samples. Keratinocytes and fibroblasts were isolated and cultured as described in Pontiggia et al. [10], and melanocytes as specified in Böttcher-Haberzeth et al. [11].

Preparation of pigmentDESS

PigmentDESS were prepared as described in Biedermann et al. [12]. In brief, as a dermal portion, collagen type I was casted in 6-well cell culture inserts with 3.0 μm pore-size membranes (BD Falcon, Switzerland). Rat tail collagen type I (0.7 ml) (BD Biosciences, Switzerland) was mixed with 0.2 ml chilled neutralization buffer containing 0.15 M NaOH and 1×10^5 fibroblasts (passage 2). After gel polymerization (10 min at room temperature, and 20 min at 37 °C), the dermal equivalents were cultured in DMEM/10 %FCS (Invitrogen, Switzerland) for 5 days. Subsequently, human keratinocytes and melanocytes (ratio 5:1, passages 2–3) were seeded onto the dermal equivalent at a density of 5×10^5 cells. The pigmentDESS were cultured in SFM (Invitrogen, Switzerland) for 1 week before transplantation. The medium was changed every second day.

Transplantation of cultured pigmentDESS

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 76/2011). Immuno-incompetent female nu/nu rats, 8–10 weeks old (Charles River, Germany), were prepared and anesthetized as previously described [13]. As protection of the pigmentDESS and as prevention from wound closure by the rat skin, custom-made surgical steel rings (diameter 2.6 cm) were sutured into full-thickness skin defects created on the backs of the rats, using non-absorbable polyester sutures (Ethibond®, Ethicon, USA). The transplants were then covered with a silicone foil (Silon-SES, BMS, USA), a polyurethane sponge (Ligasano, Ligamed, Austria), and a tape (Leukoplast, BSN medical, Germany) as wound dressing. Dressing changes were performed once per week.

Analyses of transplanted pigmentDESS

The pigmentDESS were excised 15 weeks after transplantation and processed for paraffin or cryo-embedding.

Immunohistochemical analysis was performed as described by Kiowski et al. [14]. Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated. For antigen retrieval, slides were heated with cell conditioner 1 (Ventana Medical Systems, Tucson, AZ). Sections were stained with the following antibodies: MITF (clone 5þD5, 1:50, Abcam, Germany) and SOX10 (clone N-20, 1:50, Santa Cruz, USA). The antibody binding was visualized using detection kits (iVIEW DAB detection and ultraView Universal Alkaline Phosphatase Red Detection kits, Ventana, Switzerland). Slides were counterstained with hematoxylin.

Cryosections were stained according to Biedermann et al. [15]. The following antibodies were used: HMB45, (clone HMB45, 1:50, Dako, Germany), BCL2, 1:50, clone

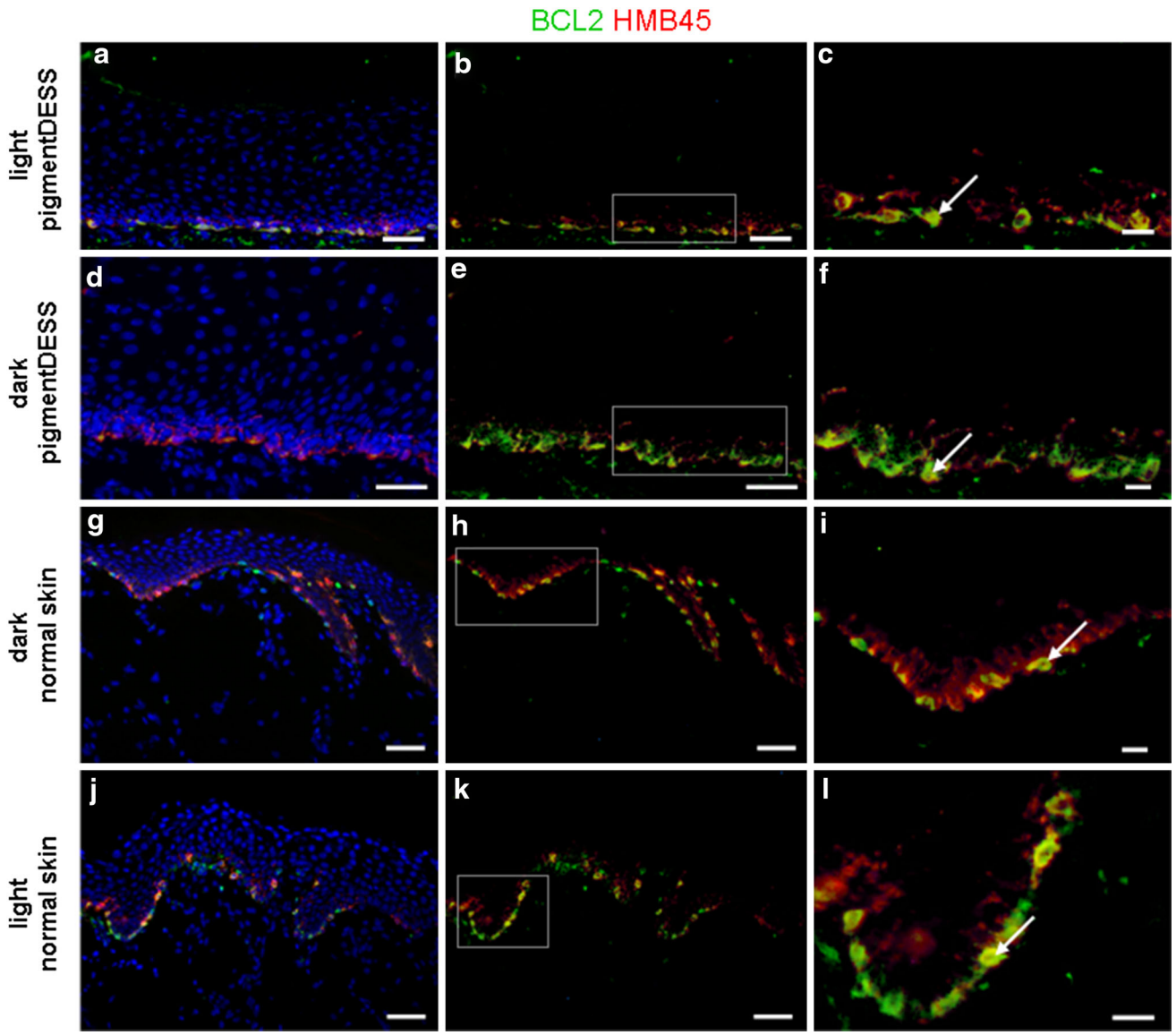

Fig. 1 Expression pattern of BCL2 in pigmentDESS and normal human skin. Expression of BCL2 (*green*) and HMB45 (*red*) in light pigmentDESS 15 weeks after transplantation. **a** Expression of BCL2 is restricted to the basal layer of the epidermis. **b** Expression pattern without a nuclei counterstaining for better overview. **c** A magnification of the *white dotted square* in **b** highlighting the co-expression of BCL2 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of BCL2 (*green*) and HMB45 (*red*) in dark pigmentDESS 15 weeks after transplantation **d** with and **e** without nuclear counterstaining. **f** Highlighting the co-expression of BCL2 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of BCL2 (*green*) and HMB45 (*red*) in dark human skin **g** with and **h** without nuclear counterstaining. **i** Highlighting the co-expression of BCL2 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of BCL2 (*green*) and HMB45 (*red*) in light human skin **j** with and **k** without nuclear counterstaining. **l** Highlighting the co-expression of BCL2 and HMB45 (*white arrow*) in epidermal melanocytes. Cell nuclei are stained with Hoechst (*blue*). *Scale bars* **a**, **b**, **d**, **e**, **g**, **h**, **j**, **k** 100 μm and **c**, **f**, **i**, **l** 20 μm

124, Abnova, Germany), SOX9 (clone 3C10, 1:50, Abcam, Germany).

Pictures of immunofluorescence stainings were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. The device is equipped with Hoechst 33342, FITC, and TRITC filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 vers. 2.70). Images were processed with Photoshop 11.0 (Adobe Systems Inc, Germany).

Results

Expression of BCL2 in transplanted pigmentDESS

The pigmentDESS were analyzed 15 weeks after transplantation for the presence and the expression pattern of the mitochondrial anti-apoptotic marker BCL2 in the epidermis. In light and dark pigmentDESS, BCL2 (green) was expressed in a comparable manner (Fig. 1a–f). In both

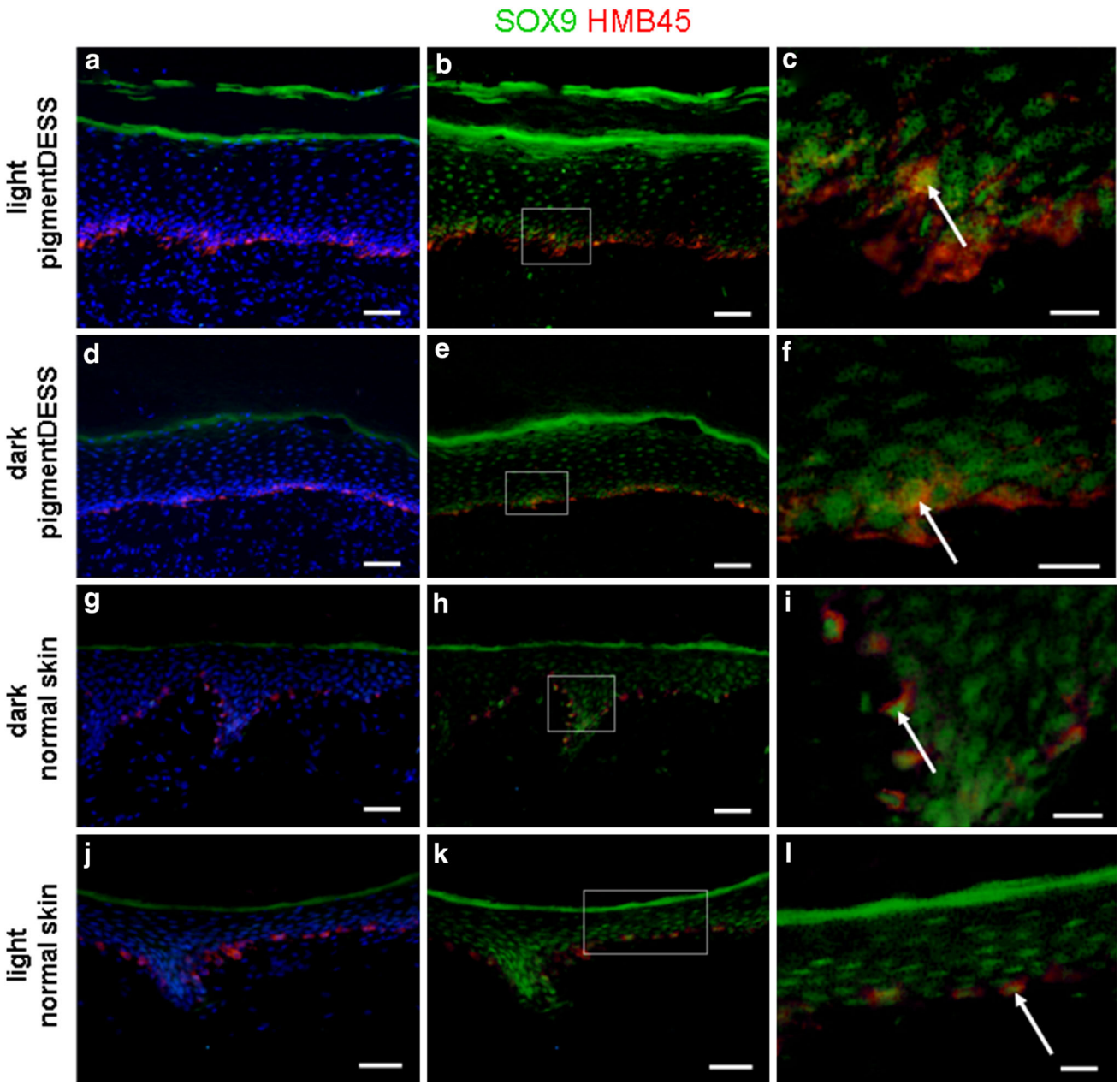

Fig. 2 Expression pattern of SOX9 in pigmentDESS and normal human skin. Expression of SOX9 (*green*) and HMB45 (*red*) in light pigmentDESS 15 weeks after transplantation. **a** Expression of HMB45 (*red*) is seen in melanocytes of the basal layer of the epidermis. **b** Displaying the expression pattern without a nuclei counterstaining showing the expression of SOX9 in keratinocytes in all layers of the epidermis and in melanocytes in the basal layer of the epidermis. **c** is a magnification of the *white dotted square* in **b** highlighting the co-expression of SOX9 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of SOX9 (*green*) and HMB45 (*red*) in dark pigmentDESS 15 weeks after transplantation **d** with and **e** without nuclear counterstaining. **f** Highlighting the co-expression of SOX9 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of SOX9 (*green*) and HMB45 (*red*) in dark human skin **g** with and **h** without nuclear counterstaining. **i** Highlighting the co-expression of SOX9 and HMB45 (*white arrow*) in epidermal melanocytes. Expression of SOX9 (*green*) and HMB45 (*red*) in light human skin **j** with and **k** without nuclear counterstaining. **l** Highlighting the co-expression of SOX9 and HMB45 (*white arrow*) in epidermal melanocytes. Cell nuclei are stained with Hoechst (*blue*). *Scale bars a*, **b**, **d**, **e**, **g**, **h**, **j**, **k** 100 μm and **c**, **f**, **i**, **l** 20 μm

types of DESS, the BCL2 expression was restricted to the basal layer of the epidermis. To confirm the presence of BCL2 in epidermal melanocytes, a staining against the melanocyte-specific marker HMB45 (red, Fig. 1a–f) was applied. The double staining without cell nuclei counterstaining for light (Fig. 1b) and dark (Fig. 1e) pigmentDESS

is shown for better overview. The white arrows in Fig. 1c and f (higher magnifications of the white squares in Fig. 1b and e) indicate melanocytes double positive for BCL2 and HMB45.

For comparison, dark and light normal human skin was stained for BCL2 (green) and HMB45 (red) (Fig. 1g–l). BCL2-expressing cells were detected in the basal layer of the epidermis in dark and light skin (Fig. 1h, k). In a higher magnification, BCL2 and HMB45 double-positive melanocytes are shown (Fig. 1i, l, white arrows).

Expression of SOX9 in transplanted pigmentDESS

The transplanted pigmentDESS were also analyzed regarding the presence and expression pattern of the nuclear transcription factor SOX9 in the epidermis. In light and dark pigmentDESS, SOX9 (green) was expressed throughout the cells in the epidermis in a comparable manner (Fig. 2a–f). To confirm the presence of SOX9 in melanocytes, the double staining with HMB45 and without cell nuclei counterstaining for light (Fig. 2b) and dark (Fig. 2e) pigmentDESS is shown for better overview. A higher magnification thereof is shown in Fig. 2c and f; white arrows point at melanocytes double positive for SOX9 and HMB45, confirming the expression of SOX9 in melanocytes.

Dark and light normal human skin was stained for SOX9 and HMB45 (Fig. 2g–l) for comparison. SOX9 was expressed in all the cells of the epidermis in dark and light skin. In a higher magnification, SOX9 and HMB45 double-positive melanocytes are shown (Fig. 2i, l, white arrows).

Expression of SOX10 and MITF in transplanted pigmentDESS

Immunohistochemical stainings against the transcriptional factors SOX10 (Fig. 3) and MITF (Fig. 4) were used to determine the expression of these two melanocyte markers in pigmentDESS 15 weeks after transplantation.

SOX10 is expressed in the nuclei of melanocytes in the basal layer of the epidermis in light (Fig. 3a, black arrows) and dark (Fig. 3b, black arrows) pigmentDESS. MITF expression was also detected in the nuclei of melanocytes in the basal layer of the epidermis in light (Fig. 4a, black arrows) and dark (Fig. 4b, black arrows) pigmentDESS.

Discussion

In our experimental study, we investigated if dermo-epidermal skin substitutes containing melanocytes that were transplanted express melanocyte-specific markers in the long run (15 weeks after transplantation). In general, we observed a similar pattern of marker expression in these pigmentDESS as in normal human skin. Several aspects of our findings deserve a detailed comment.

We have previously shown that we can tissue engineer human light and dark dermo-epidermal skin substitutes with a color that is almost identical to one of the donor skins independently of the used melanocyte:keratinocyte ratio [9]. Here, we used the described physiological melanocyte:keratinocyte ratio of about 1:5 in the basal layer of the epidermis to prepare the light or dark pigmentDESS that were then transplanted [16–18].

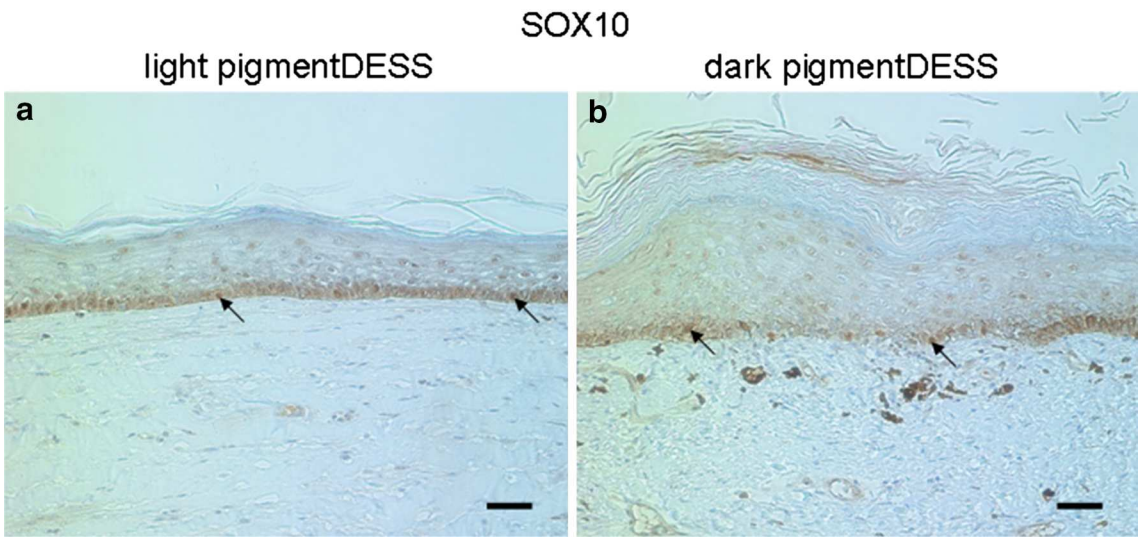

Fig. 3 Expression of SOX10 in pigmentDESS 15 weeks after transplantation. **a** SOX10 staining in light pigmentDESS and **b** staining in dark pigmentDESS. SOX10 expression is restricted to melanocytes in the basal layer of the epidermis (*black arrows*). *Scale bars* 50 µm

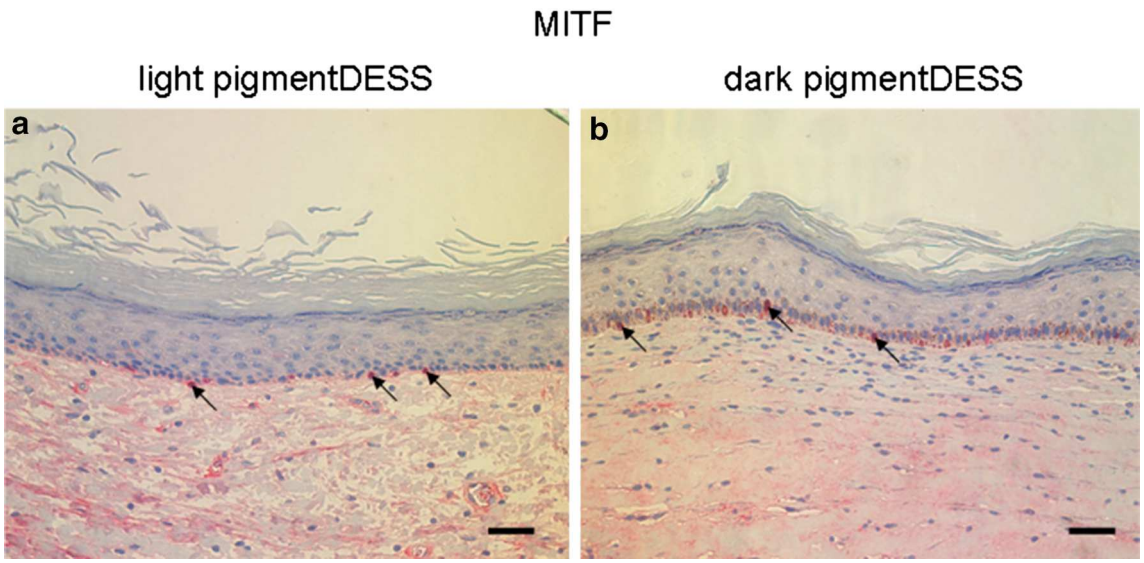

Fig. 4 Expression of MITF in pigmentDESS 15 weeks after transplantation. **a** MITF staining in light pigmentDESS and **b** staining in dark pigmentDESS. MITF expression is restricted to melanocytes in the basal layer of the epidermis (*black arrows*). *Scale bars* 50 μm

First, we investigated the presence of a well-described anti-apoptotic cell marker, namely BCL2. BCL2 is one of the major members of the BCL2 family of anti-apoptotic proteins that includes also BCL-xL and MCL-1 [19]. These proteins are generally situated in the outer mitochondrial membrane in the cells. There they bind and inhibit the pro-apoptotic members of the BCL2 family as, e.g., BAK and BAX, so preserving the outer mitochondrial membrane stability and preventing cell apoptosis by circumventing the intrinsic apoptotic pathway [20]. Studies describe the presence of BCL2 in fetal and adult human skin, with a higher level of BCL2 protein expression in melanocytes than in keratinocytes [21–23]. This higher level of BCL2 in melanocytes is thought to impede UV irradiation-induced apoptosis, whereas keratinocytes which contain less BCL2 are more susceptible for apoptosis already at low doses of UV irradiation [24].

We observed the presence of BCL2 in melanocytes of pigmentDESS 14 weeks after transplantation basically reflecting the physiological situation of normal human skin. This suggests that these melanocytes are physiologically functioning and might efficiently protect against solar radiation which is of pivotal importance, once pigmentDESS are applied clinically.

Second, we analyzed the expression of the transcription factors SOX9 and SOX10 in pigmentDESS melanocytes. Lately, it was observed that SOX9 drives the transition of undifferentiated melanoblasts to differentiated melanocytes in postnatal skin eventually resulting in melanogenesis, i.e., the production of melanin [25–27]. SOX10 is a widely described transcription factor and one of the most important regulators of embryonic melanocyte development and of maintenance of melanocyte stem cells in hair follicle bulge in postnatal skin [28, 29]. Furthermore, SOX10 is expressed in postnatal melanocytes of the epidermal basal layer, keeping them in a more undifferentiated state [30]. Both SOX9 and SOX10 activate the expression of the transcription factor MITF [31]. MITF itself is a well-characterized master regulator of melanocyte survival and melanogenesis, thereby regulating the expression of tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrp1), and 2 (Tyrp2) that is crucial for melanin synthesis [32, 33].

In our pigmentDESS, we observed the presence of SOX9 in melanocytes in the basal layer of the epidermis and, moreover, also some SOX10 positive melanocytes. As MITF expression is activated by SOX9 and 10, the presence of MITF in melanocytes of our transplanted pigmentDESS does not surprise. It demonstrates that another functional pathway, namely the induction of MITF by SOX9 and 10 seems to work in a physiological way.

Taken together, our molecular findings regarding expression of transcription factors regulating melanocyte differentiation and melanogenesis (the pigment production) are in line with our previous macroscopic observations that transplanted light and dark pigmentDESS display the same color as the donor skin color [9].

One could speculate that, because of the presence of SOX10, undifferentiated melanocytes, even melanocyte precursor cells, are residing in the epidermis of our pigmentDESS. This leads to the assumption that not only 15 weeks after transplantation but also for an indefinite longer time, melanocyte precursors (that give rise to melanocytes) could be maintained in our dermo-epidermal skin

substitutes, which is again of clinical importance, as we want to produce pigmentDESS that guarantee the patients' skin color for life.

In future studies, we could use our model to analyze the impact of UV light onto pigmentDESS, and look at the presence and change of marker expressions after UV irradiation. It was described previously that BCL2 was upregulated in melanocytes through MITF activation after UV irradiation and thereby enhancing melanocyte survival [23, 24]. In addition, human melanocytes isolated and grown in vitro upregulated SOX9 after UV irradiation and increased their melanin production [26].

In conclusion, we observed that our pigmentDESS express typical melanocyte survival, differentiation, and melanogenesis markers 15 weeks after transplantation. These findings have significant implications regarding the envisioned clinical application.

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Conflict of interest The authors declare that they have no conflict of interest.

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